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MECHANISMS INFLUENCING NATURAL KILLER CELL RECOGNITION OF TUMOUR CELLS

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ABSTRACT

Natural killer (NK) cells are part of our innate immune defence against virus-infected and transformed cells. Through a yet undefined mechanism, expression of inhibitory receptors for self-HLA class I molecules endow NK cells with increased functionality. The process leading to gain of function through inhibition is known as NK cell education. In the first part of this thesis the cellular mechanisms behind this phenomenon were studied. We found that the expression levels of the activation/adhesion molecule DNAM-1 correlated with the education state in NK cells. Our results suggest that DNAM-1 together with coordinated conformational changes in the adhesion molecule LFA-1 may contribute to the heightened effector functions in educated NK cells.

A decreased NK cell function has been previously associated with impaired immune surveillance and a higher risk for developing cancer. By studying samples from patients diagnosed with myelodysplastic syndromes (MDS), we found that NK cells in the bone marrow were phenotypically altered and functionally impaired. Two activating receptors, DNAM-1 and NKG2D were decreased, and the ability to recognise and kill MDS blast cells compromised. The phenotypic alterations correlated with the frequency of leukemic blast cells suggesting that the immune dysfunction progress with the severity of the disease.

In one part of this thesis possibilities to improve tumour cell recognition by NK cells were examined. Oxidative stress induced by selenite in a tumour cell line lowered the surface expression of HLA-E, which is a ligand for inhibitory NKG2A receptors expressed by a large proportion of human NK cells. The downregulation of HLA-E led to increased NK cell detection and killing of the tumour cells by $NKG2A^+$ NK cells.

Therapeutic antibodies have led to a paradigm shift in the care of patients with malignant lymphoma. Yet the mechanisms of action and the contribution of discrete immune subsets to the clinical efficacy are largely unknown. One suggested mechanism is NK cell mediated antibody-dependent cellular cytotoxicity. We studied NK cell repertoires in sequential lymph node biopsies from follicular lymphoma patients undergoing treatment with anti-CD20 antibodies. After treatment there was a decrease in peripheral NK cells, and the remaining NK cells from both peripheral blood and tumour-associated lymph nodes were activated. Furthermore, the NK cells showed an altered ability to produce cytokines after *in vitro* re-stimulation. In conclusion, the data presented here provide new insights into how NK cells recognise and respond to tumour cells at steady state and in malignant diseases.

LIST OF SCIENTIFIC PAPERS

This thesis is based on three publications and one manuscript. The individual papers are referred to by roman numerals.

I. Coordinated expression of DNAM-1 and LFA-1 in educated NK cells.
Monika Enqvist, Eivind Heggernes Ask, Elin Forslund, Mattias Carlsten, Greger Abrahamsen, Vivien Béziat, Sandra Andersson, Marie Schaffer, Anne Spurkland, Yenan Bryceson, Björn Önfelt and Karl-Johan Malmberg
The Journal of Immunology. 2015, 194: 4518–4527

II. Reduced DNAM-1 expression on bone marrow NK cells associated with impaired killing of $CD34^+$ blasts in myelodysplastic syndrome.
Mattias Carlsten, Bettina C Baumann, **Monika Simonsson**, Martin Jädersten, Ann-Marie Forsblom, Christina Hammarstedt, Yenan T Bryceson, Hans-Gustaf Ljunggren, Eva Hellström-Lindberg and Karl-Johan Malmberg
Leukemia. 2010, 24, 1607–1616

III. Selenite induces posttranscriptional blockade of HLA-E expression and sensitizes tumor cells to CD94/NKG2A-positive NK cells.
Monika Enqvist, Gustav Nilsonne, Oscar Hammarfjord, Robert PA Wallin, Niklas K Björkström, Mikael Björnstedt, Anders Hjerpe, Hans-Gustaf Ljunggren, Katalin Dobra, Karl-Johan Malmberg and Mattias Carlsten
The Journal of Immunology. 2011, 187: 3546–3554

IV. Systemic and intra-nodal activation of NK cells after rituximab treatment in patients with follicular lymphoma.
Monika Enqvist, Henna R Junlén, Benedikt Jakobs, Marie Schaffer, Christopher M Melén, Danielle Friberg, Björn E Wahlin and Karl-Johan Malmberg
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependant cellular cytotoxicity
AML	Acute myeloid leukaemia
CD	Cluster of differentiation
CMV	Cytomegalovirus
CXCR	C-X-C chemokine receptor
DAP	DNAX adaptor protein
DC	Dendritic cell
DNAM	DNAX adaptor molecule
FL	Follicular lymphoma
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immune tyrosine-based activation motif
ITIM	Immune tyrosine-based inhibitory motif
KIR	Killer cell immunoglobulin-like receptor
LFA	Lymphocyte function-associated antigen
LN	Lymph node
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MIC	Major histocompatibility complex class I-related chain
MIP	Macrophage inflammatory protein
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKG	Natural killer group
PB	Peripheral blood
PVR	Poliovirus receptor
ROS	Reactive oxygen species
SH2	Src homology 2

STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	T regulatory cell
ULBP	UL16 binding protein
WT	Wildtype

1 INTRODUCTION

The immune system constitute of cells and molecules protecting our body against uncountable threats, including viruses, bacteria and sometimes our own cells behaving in an inappropriate way. The immune system can be divided into two arms, the innate and the adaptive arm. Components of the innate immune system respond broadly and rapidly to potentially dangerous challenges. The adaptive immune system is characterised by cells and effector molecules that mount a delayed, yet more specific response towards a pathogen, which involves development of a long-lasting memory so that our immune system can respond more efficiently to a second challenge. The human natural killer (NK) cell is a cell-type traditionally considered part of the innate immune system, and is the main focus of this thesis.

1.1 NATURAL KILLER CELLS

NK cells are a type of white blood cells known for their ability to eliminate virus-infected or malignant cells and to secrete cytokines. The name 'Natural Killer' refers to the fact that these cells are ready to act and kill potential threats directly and without prior sensitisation (1, 2). NK cells are found in our blood at frequencies around 5-15% of total lymphocytes, but also at varying numbers in other organs as in the bone marrow, secondary lymphoid organs, liver and uterus (3).

1.1.1 Differentiation

NK cells originate from hematopoietic stem cells (4). The stem cells differentiate into common lymphoid progenitors with maintained potential to become T cells, B cells or a variety of innate lymphoid cells (ILCs) (5). The lack of antigen specific receptors separates ILCs from the adaptive T- and B-lymphocytes (5). There are three main groups of ILCs described, with NK cells belonging to type 1 ILCs. (6) Cells in this group share features as expression of the transcription factors T-bet and Eomes, and effector functions as cytotoxicity and interferon (IFN)-γ cytokine production (7). Apart from NK cells, the different types of human ILCs have only recently been characterised, and the strict division between a human NK lineage progenitor and other closely related innate lymphoid cells is not clear (8).

NK cells mature in the bone marrow, but precursors with ability to differentiate to mature NK cells have been found also in other organs as liver, tonsils and thymus (4, 9-13). Interleukin (IL)-15 is an essential cytokine for NK cell development, proliferation and survival (14). NK cells in peripheral blood are defined as CD3 negative and positive for the cell surface molecule CD56, expressed at either high ($CD56^{bright}$) or medium ($CD56^{dim}$) levels. It is not clear if $CD56^{bright}$ and $CD56^{dim}$ NK cells represent two different lineages, as supported by studies of haematopoiesis in macaques (15), or if $CD56^{bright}$ are the precursors of $CD56^{dim}$ cells. The $CD56^{bright}$ cells have shorter telomeres than $CD56^{dim}$ cells (16), and can acquire phenotypic similarities to $CD56^{dim}$ NK cells *in vitro* and in humanised mice (17, 18). $CD56^{bright}$ cells respond primarily to cytokine stimulation and are efficient cytokine producers

(19). Recently, it has become clear that the $CD56^{dim}$ subset is highly diversified in part through a continuous differentiation process (17, 20-22). The least mature cells have more similarities to the $CD56^{bright}$ cells and express higher level of cytokine receptors, NKG2A and natural cytotoxicity receptors (NCRs), and have a high proliferative capacity. The maturation process includes a loss of cytokine responsiveness, and acquisition of inhibitory killer cell immunoglobulin receptors and CD57, a cell surface marker correlated with senescence in T cells (23). Additionally, there is an increase of molecules associated with cellular cytotoxicity, as CD16, granzyme B and perforin (22). The most mature NK cell subsets are highly cytotoxic and efficient cytokine producers in response to activating receptor stimulation, including via antibody-coated cells (22, 24).

Based on the cell surface receptor expression profile, the NK cell population is very heterogeneous, yet stable over time (25). One lasting influence on the NK cell repertoire is an infection with cytomegalovirus (CMV), which is correlated with an increased level of NK cells expressing an activating receptor CD94/NKG2C (25-32). CMV infection induces expansion of a memory NK cell subset in mice (33). This subset has an activating receptor, Ly49H that specifically recognise a mouse CMV-encoded peptide expressed on infected cells (33). Such antigen-specificity has not been found in human NK cells, although recent studies have shown NK cell subsets exhibiting adaptive immune features including stable epigenetic modifications (34-37).

1.1.2 Receptors

NK cells express numerous germline-encoded receptors on the cell surface, some are summarised in table 1.

Inhibitory receptors

The two major families of inhibitory NK cell receptors in humans are the heterodimer CD94/NKG2A and inhibitory killer cell immunoglobulin-like receptors (KIRs). CD94/NKG2A belongs to the C-type lectin receptors, and binds to HLA-E (38, 39). KIRs bind to specific allelic variants of HLA-A, -B and –C, encoding human major histocompatibility (MHC) class I molecules (40). As with the *MHC class I* gene locus, the *KIR* gene locus is highly polymorphic and on a population level numerous combinations of *KIRs* and allelic variants exists (41). Additionally, the KIR expression on individual NK cells differs despite identical gene content (42).

The cytoplasmic domain of the inhibitory receptors contains immunoreceptor tyrosine-based inhibition motifs (ITIMs) (43). Upon binding to its HLA ligand the inhibitory signal is transmitted via ITIM phosphorylation and recruitment of phosphotyrosine or inositol phosphatases, which in turn can lead to decreased tyrosine phosphorylation of effectors in activation pathways (44). The inhibition prevents Ca^{2+} influx, actin rearrangements and formation of lipid rafts (45, 46).

Table 1: NK cell receptors and their ligands

Receptor	Ligand	Function
CD94/NKG2A	HLA-E	Inhibitory
KIR2DL1	HLA-C2	Inhibitory
KIR2DL2/3	HLA-C1	Inhibitory
KIR3DL1	HLA- Bw4	Inhibitory
KIR3DL2	HLA-A11/A3	Inhibitory
KIR2DS1	HLA-C2	Activating
KIR2DS2		Activating
KIR3DS1		Activating
CD16	IgG	Activating
CD94/NKG2C	HLA-E	Activating
NKG2D	ULBP, MICA/B	Co-activating
NKp30	B7-H6	Co-activating
NKp44		Co-activating
NKp46		Co-activating
2B4	CD48	Co-activating
DNAM-1	CD155, CD112	Co-activating, adhesion
LFA-1	ICAM-1	Adhesion, granule polarisation

Activating receptors

Activating NK cell receptors generally require combinatory engagement in order to induce a cytotoxic or cytokine response to take place (47, 48). An exception is engagement of the low-affinity Fc-receptor CD16, which binds antibody on target cells and is sufficient to induce NK cell degranulation (49). Activating receptors transmit the signals via cytoplasmic adaptor proteins and various signalling domain and pathways exists (50).

CD16 associates with FcεRγ and CD3ζ adaptors, both carrying immunoreceptor tyrosine-based activation motifs (ITAMs) (50). Receptor ligation leads to phosphorylation of the ITAMs by Src family-kinases, and engagement of SH2-domain containing signalling proteins that propagate activating signals (51).

NKG2D is an activating receptor that binds to several ligands, including MICA, MICB and the ULBP family (52). These ligands are up-regulated on virus-infected cells, DNA damaged or transformed cells (53). Mice deficient in NKG2D have an increased tumour outgrowth, suggesting an important role for this receptor in tumour surveillance and elimination (54).

NKG2D associates with a DAP10 adaptor signalling protein, which has a tyrosine-based motif that can be phosphorylated upon activation for signalling transduction (55).

DNAM-1, also known as CD226, is an activating and adhesion molecule (56). Similar to NKG2D it binds to stress-induced ligands, for DNAM-1 it is CD155 (PVR) and CD112 (Nectin 2) (57). These ligands are commonly expressed at high levels on tumour cells, and DNAM-1 has been shown to have an essential part in immune surveillance (58-62). CD155 is also expressed on dendritic cells (DCs) and T cells, and the DNAM-1/CD155 interaction has been proposed to play a role in selective NK-cell mediated DC-editing during an immune response (63). The intracellular signalling downstream of DNAM-1 is not known, but the receptor can functionally and physically associate with lymphocyte function-associated antigen-1 (LFA-1) (64).

Adhesion molecules

Adhesion molecules are important for NK cell trafficking to tissues as well as initiation and promotion of contact with target cells. One essential molecule in formation of conjugates between cells is LFA-1 (65). LFA-1 binds to intracellular adhesion-molecule-1 (ICAM-1), and this alone is sufficient in NK cells to induce an activating signal and polarise granules (49, 66). Stimulation through activating receptors leads to an inside-out signal to LFA-1 (67). This changes the conformation of LFA-1 to an open, active form that facilitates stable conjugate to be formed with ICAM-1 expressing cells (68). NK cells, in contrast to T cells (69), do not need an inside-out-signal to activate LFA-1, although the signal is required for further stable conjugate propagation, activation and granular release (50).

1.1.3 Activation

The stimulation of NK cells through activating receptors or cytokines can lead to cytotoxic response and production and release of cytokines and chemokines as IFN-γ, TNF-α and MIP-1β (70). In contact with target cells, NK cells can form an immunological synapse with activating molecules clusters in the central part and adhesion molecules as LFA-1 in the peripheral part (71). Inhibitory and cytotoxic synapses have been shown to be formed in the same way in NK cells and differ in the ratio between activating tyrosine kinases and the tyrosine phosphatases in the central part of the conjugate (72-74). A high ratio will through signalling cascades ultimately lead to Ca^{2+} influx, re-organisation of actin cytoskeleton and delivery of lytic granules to the synapse via microtubule transport. The granules released in the cytotoxic synapse contain perforin, a pore forming protein, and granzymes, proteins capable of inducing apoptosis once entering the target cell (75). NK cells can furthermore upon ligation of 'death receptors', TRAIL and Fas-ligand, induce apoptosis in target cells (76).

NK cells have receptors for type I interferons, and can be activated by the release of pro-inflammatory cytokines as IL-12, IL-18 and TNF (77, 78). Signalling through cytokine receptors can lead to STAT phosphorylation and transcription, production and secretion of IFN-γ, an important mediator of pro-inflammatory and antiviral effects (79). The signalling

pathways for cytokine production and induction of cytotoxic responses are overlapping, and both cytokine activation of NK cells and stimulation through activating receptors can promote a cytotoxic response and induce cytokine production. (80)

1.1.4 Functional regulation

Missing-self-recognition

The functional regulation of NK cells was in the 1980's hypothesised as a missing-self recognition (81). In contrast to T cells, where activation occurs upon recognition of HLA class I molecules presenting non-self peptides, NK cells could detect and respond to cells that down-regulated or lacked expression of self-HLA class I molecules (82). Predictions made in the hypothesis were confirmed when the inhibitory KIRs in humans and Ly49 receptors in mice were identified and shown to bind self-expressed MHC class I molecules (83, 84). In addition to lack of inhibition, NK cells require activation signals to trigger a functional response (47).

Education

A more recently described role for inhibitory receptors is to tune the threshold for activation and determine the functional capacity of the cell in a process referred to as 'education' (85). In order to develop highly efficient effector functions NK cells need to express at least one type of inhibitory receptor recognising self-expressed HLA molecules. It remains unknown how the inhibitory signal in NK cells is translated to an increased functional competence in terms of cytotoxicity and cytokine production upon activation. Several models to explain NK cell education have been proposed. In the disarming model all cells are initially fully competent and require the inhibitory input to maintain their functionality (86, 87). In the arming model, cells acquire the increased effector function along with self-inhibitory receptors during development (88, 89). Compatible with both models, more recent data suggests that education is not an on and off switch (90). Instead the NK cell function can be likened to a rheostat, being adjusted depending on the MHC class I environment and the amount of inhibitory interactions the cell receives (90).

1.2 TUMOUR CELLS

A normal cell can transform into a tumour cell and ultimately cause severe damage to the body. For this to occur the shared hallmarks described are; sustained proliferative capacity, evasion of growth suppressors, induction of angiogenesis, activating invasion and metastasis, enabling of replicative immortality, resistance to cell death, reprogrammed energy metabolism and evasion of immune destruction (91). Tumour cells are an enormously heterogeneous population and this section will briefly introduce two human diseases of particular relevance for paper II and IV.

1.2.1 Myelodysplastic syndromes

Myelodysplastic syndromes (MDS) are a group of hematopoietic stem cell disorders in the bone marrow (92). Mainly older adults are diagnosed with MDS and median age at diagnosis

is 76 years (93). The cause of MDS is mainly unknown, but in approximately 15% of the cases it is associated to earlier use of radiotherapy, chemotherapy, tobacco smoking or exposure to benzene. (92) The disease is characterised by ineffective haematopoiesis and cytopenias as a consequence from increased apoptosis in myeloid progenitors (94). One third of the MDS patients progress to acute myeloid leukemia (AML), with high frequencies of myeloblasts (>20%) (95). A shift from apoptosis to proliferation in the progenitors may be one factor that result in progression to AML (96, 97). One type of MDS is the 5q syndrome, where a variable number of genes have been deleted on the 5q chromosome (98). This can lead to blocked erythroid proliferation due to reduced gene expression of *RPS14* (99, 100), and other defects reflecting the loss of genes.

The cell expansion in MDS is often oligoclonal with several different types of gene mutations contributing to the pathogenesis (92). These mutations are frequently affecting genomic stability and gene expression patterns and include mutations in transcription factors, epigenetic regulators involved in methylation of cytosines or histone modifications (101). Some of the most recurrently mutated genes are: *TP53*, *ETV6*, *DNMT3A*, *TET2*, *IDH1*, *IDH2*, *EZH2*, *UTX*, *ASXL1* (92). Other frequently mutated genes encode parts of the splicing machinery, which may have several functional consequences including disruption of genomic stability (102, 103). Somatic mutations are of prognostic value and mutations in *TP53*, *ETV6*, *RUNX1* or *EZH2* effects survival and worsen the prognosis (104, 105). Some epigenetic changes as aberrant methylation of gene promoters have been seen in MDS (92). Furthermore, the microenvironment and immune cells may contribute to the disease progression and increased numbers of T regulatory cells (Tregs) have been seen in patients with high-risk MDS (106).

The treatment of MDS is primarily dependent on disease severity (92). Treating high-risk patients with a median survival of 12 months aim to prolong survival, avoid AML and modify the disease progression. The group of patients with low or intermediate risk is treated mainly to improve the cytopenia as well as quality of life, since many of these patients will not die from their MDS. Lower-risk MDS patients generally receive erythropoiesis-stimulating agents, but many will eventually though need erythrocyte transfusions. For high-risk MDS, the only cure is allogeneic stem-cell transplantation. However, there is a high risk of transplant-related mortality and only a few patients can be offered this treatment. Chemotherapy, similar to treatment for AML, can be suitable for patients younger than 65 years and with favourable cytogenetics, sometimes as pre-treatment to transplantation. Clinical trials have found a survival benefit and delayed progression to AML using azacitidine, a hypomethylating agent in high-risk MDS, compared to supportive care or chemotherapy (107). However, even if the median survival is prolonged with azacitidine it is still only 2 years, and several combinations of drugs are currently being examined (92).

1.2.2 Follicular lymphoma

Follicular lymphoma (FL) is a B cell disease originating in lymph node germinal centres (108). A germinal centre in FL resembles a healthy follicle, with follicular DCs, macrophages

and T cells supporting the growth of B cells (109). 90% of all follicular lymphomas have a chromosomal translocation t(14;18), leading to over-expression of an anti-apoptotic Bcl-2 protein (110, 111). Increased Bcl-2 expression gives the lymphoma cells a survival advantage, but similar to normal B cells in the germinal centre, they are dependent on surrounding cells for development (112). The FL progression towards additional genetic abnormalities might be facilitated by excessive Bcl-2 protein expression (113). Another alteration, observed in 89% of FL's is a mutation in *MLL2*, a gene encoding a histone methyltransferase (114). This can impair cell transcription but the exact consequences of this are still unknown. Studies have shown that an increase of $CD8^+$ T cell infiltrate in FL correlates to better prognosis (115, 116). Furthermore, gene expression profiling revealed that mostly T cell related genes were linked with favourable prognosis, while genes expressed by macrophages were associated with poor prognosis (117). The composition of cells in the tumour environment is thought to have a prognostic role, but many studies have so far shown inconsistent results, possibly due to different treatment protocols (117).

Since 1998, a cornerstone of FL therapy is rituximab, a monoclonal antibody recognising the CD20 antigen expressed on mature B cells (118). This is administrated alone or in combination with chemotherapy (119). Most patients respond well, but the relapse rate is high and the disease considered incurable (120). Furthermore, 25% of the patients experience transformation of the disease to a more aggressive form of B cell lymphoma, requiring intense chemotherapy (121). The median survival in FL is 13 years (122).

The use of monoclonal antibodies as rituximab can have several effects (123). Binding of CD20 itself to malignant B cell lines is sufficient to induce cell death *in vitro* (124). Other *in vitro* studies have shown complement-dependent cytotoxicity for rituximab (125, 126), although the role of components from the complement system within lymph nodes is unknown. The third mechanism of action for rituximab is through ADCC mediated by NK cells, and/or antibody-dependent cellular phagocytosis mediated by macrophages (127, 128). NK cells engage the CD16 receptor to the Fc-part of antibody bound to target cells. This triggers strong NK cell activation including release of cytotoxic granules and production of pro-inflammatory cytokines as IFN-γ (67). The clinical response to rituximab therapy correlates to polymorphisms in the CD16 gene, supporting a role for NK cell involvement (127, 129, 130).

1.3 CANCER IMMUNOEDITING

The immune system can shape and control cancer outgrowth. In addition, tumour cells interact with and influence their surroundings, with defective immune responses being reported in many cancer types (131). The term cancer immunoediting describes the process where the immune system prevents or promotes cancer progression (132). It is subdivided into three phases; elimination, equilibrium and the escape phase.

Originally the term cancer immunosurveillance was used to explain the protection against tumour development by the immune system (133). This hypothesis was criticised due to lack

of convincing experimental data supporting immune-mediated control of non-viral tumours. However, lessons from several different studies in mice lacking distinct immune components, including perforin, IFN, T- and B-cell receptors as well as T cells, B cells, NKT cells, γδ T cells, NK cells, monocytes and macrophages, are that the immune system do in fact control induced and spontaneously formed cancer types (134). Transferring cells from wildtype (*wt*) mice to immunocompromised mice and vice versa revealed how tumour cells were shaped (edited) by its surrounding, and the need of a broader definition for the interactions between cancer and the immune system (135, 136). Cancer immunosurveillance occurs during the elimination phase of immunoediting (137). This phase is followed by a longer equilibrium phase during which the immune system edits the phenotype of the tumour. Immune selection pressure during the equilibrium phase favours the outgrowth of non-immunogeneic escape variants during the third and final phase of immunoediting, the escape phase.

In the elimination phase, several immune effector molecules, including type I IFNs, IFN-γ, perforin, Fas-L and TRAIL have been shown to be important for protecting the host against neoplastic transformation (134). All nucleated cells can produce type I IFNs and studies using neutralising antibodies against type I IFNs or blocking type I IFN receptor have shown increased tumour growth and metastasis in mice (138). NK cells, T cells and NKT cells can produce IFN-γ (139). Similarly to type I IFNs, IFN-γ neutralising antibodies increase cancer development in mice (134). Furthermore, animals lacking a subunit of the IFN-γ-receptor or being IFN-γ-deficient, more frequently developed induced sarcoma and lymphoma compared to *wt* mice (135, 140-142). The source of IFN-γ in elimination of cancer is not known, but γδ T cells are suggested as an important producer (143). Mice lacking the activating NK cell receptors NKG2D and DNAM-1 display impaired control of tumour growth (54, 61), indicating the NK cell recognition of tumour cells is also important.

If the immune system fails to eradicate the malignant cells, the result can be outgrowth of tumour cells less recognisable by the immune cells, which are either hiding quietly or progressing to a more aggressive form. In maintaining a hidden equilibrium phase between the immune system and cancer, associations are made to the different proportions of immune cells and to the cytokine balance, where e.g. IL-12 and IFN-γ promote tumour cell elimination while IL-23 and IL-10 promote persistence (144).
The escape phase is characterised by reduced tumour cell recognition by immune cells, an increased tumour cell survival and development of an immunosuppressive environment by the tumour cells (132).

1.3.1 Tumour escape from NK cells

A decreased NK cell function has been associated with a higher risk for developing cancer (145). Moreover, dysfunctional NK cells have been described in different types of cancer diseases (146). This can be due to exhausted and desensitised NK cells, or factors secreted by the tumour cells facilitating avoidance of NK cell recognition and an impaired NK cell function (131). Inhibitory molecules produced by tumour cells can be cytokines as the transforming growth factor beta (TGF-β) (147). TGF-β can reduce NK cell activity, and this

has been linked to a reduced expression of NKp30 and NKG2D (148). Microvesicles from tumour cells can contain immunosuppressive components as microRNA and cytokines (149, 150). In AML patients such vesicles were found to contain TGF-β that modulated NKG2D expression and NK cell function (151). AML cells have in another study been shown to up-regulate CD200, a cell surface glycoprotein that interfered with NK cell function (152). Other factors affecting the NK cells are production of reactive oxygen species (ROS), and monocytic and myelo-monocytic AML cells could this way trigger NK cell apoptosis (153). Tumour cells can furthermore create a milieu promoting tumour growth while suppressing immune surveillance. Cancer cells can this way indirectly influence NK cells and NK cell function by recruitment and support of immunosuppressive cells as Tregs, type 2 macrophages and myeloid derived suppressor cells (154).

2 AIMS

I. Study cellular mechanisms underlying functional tuning of NK cells at steady state

II. Characterise the integrity of NK cell phenotype and function in patients with myelodysplastic syndromes

III. Explore possibilities to improve tumour cell recognition through modulation of the redox status

IV. Examine dynamics of NK cell repertoires and function during monoclonal antibody therapy for follicular lymphoma

3 RESULTS AND DISCUSSION

3.1 ACTIVATION AND ADHESION IN EDUCATED NK CELLS

NK cell activation is regulated by signals from activating and inhibitory cell surface receptors. Besides this, the intrinsic functional capacity of NK cells to respond varies. NK cells expressing an inhibitory receptor binding to a self-expressed HLA class I molecule have been shown able to respond stronger than cells lacking this inhibitory input, this is referred to as NK cell education or licensing (88, 155). The mechanism behind this is not fully understood and comparisons of educated and non-educated NK cells have failed to explain any major differences on a transcriptional and cellular level (155, 156). It has been suggested that the educated cells differ in organisation of receptors in the immunological synapse formed upon target interaction, which furthermore influence conjugate formation and functional responses (156, 157).

In **paper I**, we focused on activating and adhesion molecules on NK cells, finding a correlation between expression levels of the activating receptor DNAM-1 and cellular education. An earlier comparison has shown differential DNAM-1 levels on $KIR^{+}NKG2A^{+}$ versus $KIR^{-}NKG2A^{-}$ NK cells (155). Confirming and extending these results, we observed increased expression on cells having self-inhibitory KIR receptors compared to cells expressing non-self inhibitory KIRs. Additionally we found the more differentiated $CD57^{+}$ NK cells to have an amplified level of DNAM-1 and the number of educating receptors on the cell surface to correlate with DNAM-1 expression. The differentiation process in NK cells is linked to accumulation of KIRs, increased CD16 and granzyme B expression and gain of cell surface CD57, as well as an increased cytolytic capacity (21, 22). The higher level of DNAM-1 in more differentiated NK cells expressing CD57 and multiple KIRs as well as the more immature NK cells expressing NKG2A including $CD56^{bright}$ cells, suggests that the inhibitory interactions are important for maintained DNAM-1 expression on the cell surface (Figure 1).

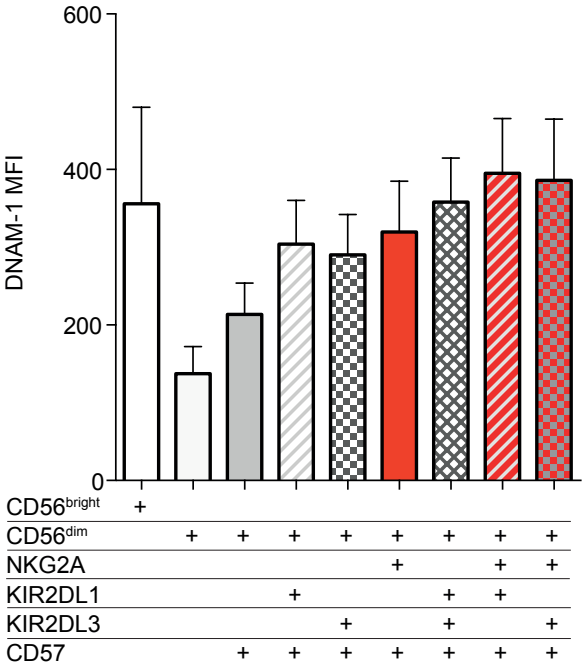

Figure 1: High DNAM-1 expression correlates with inhibitory educating KIR and NKG2A receptors. Median fluorescent intensity of DNAM-1 on the indicated NK cell subsets. Donors are $HLA\text{-}C1^{+}/C2^{+}$. The cells are negative for NKG2C, KIR3DL2, KIR2DS4, KIR3DL1 and activating KIRs. (N=16).

Previous findings have revealed down-regulation of DNAM-1 on NK cells upon physical interactions with the ligand CD155 (158). In *Cd155*-deficient mice, T cells and NK cells display increased DNAM-1 expression, which decreases if the cells are transferred to a WT mouse with normal CD155 levels (159).

CD155 is an immunoglobulin-like transmembrane protein involved in cell-cell adhesions and cell motility, and it is expressed on fibroblasts, epithelia, endothelia and hematopoietic cells including dendritic cells (DCs) and T cells (57). Interestingly DCs but not other T cells or stromal cells could modulate DNAM-1 expression *in vivo* on mouse T cells (159) and the negative selection of $CD8^+$ T cells in thymus was impaired in DNAM-1 knockouts as well as animals lacking CD155, suggesting the DNAM-1/CD155 interaction is important to obtain mature $CD8^+$ T cells (160). During NK cell maturation the contact with DCs or other $CD155^+$ cells might thus regulate the level of DNAM-1 on the NK cell surface, assuring that only cells which are competent to distinguish between self and non-self via inhibitory HLA class I-binding receptors can retain high DNAM-1 expression (Figure 2).

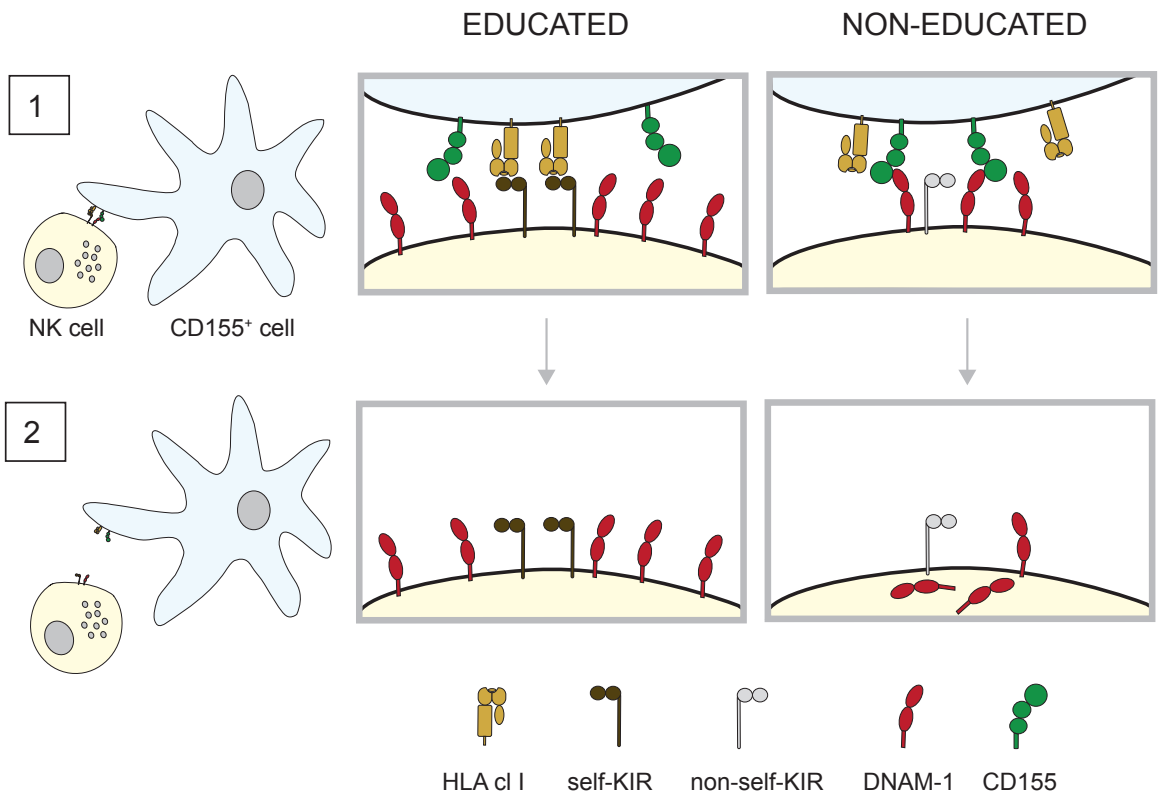

Figure 2: High DNAM-1 levels explained by inhibitory interactions. 1: Educated NK cell in contact with $CD155^+$ cell, where signalling through inhibitory receptors prevent activation. Lack of inhibition facilitates DNAM-1/CD155 binding in non-educated NK cell. 2: Cell contact ends and the educated NK cell have continued high DNAM-1 expression. The non-educated and hypo-responsive NK cell may down-regulate DNAM-1 after interaction with the ligand.

Most assays used to examine NK cell function involve activation through DNAM-1 since several common target cell lines express the ligands CD155 and CD112. Therefore it its not surprising that cells expressing higher DNAM-1 levels also respond better to stimulation with CD155/CD112^{+} target cells. In our experiments we could see a clear correlation between functional response to target cell stimulation and the DNAM-1 expression, but independently if the target cell expressed the DNAM-1 ligands or not. DNAM-1 can physically associate with the adhesion molecule LFA-1 on the cell surface (64). To find out if the LFA-1/ICAM-1 interaction was responsible for the higher response also in CD155/CD112 negative cells, we used a reductionistic model system where we co-incubated NK cells with insect cells (at baseline lacking ligands to human NK cells) transfected with specific NK cell receptor ligands. Activation of NK cells in the absence of LFA-1 and DNAM-1 ligands displayed sustained correlation of the functional response and DNAM-1 expression. This suggests the DNAM-1 expression level is an intrinsic marker for NK cells with a high capacity to preform cytotoxic and cytokine response upon contact with tumour cells.

One initial step in conjugate formation between cells is a conformational change of the LFA-1 molecule into its open, active form enabling an immune synapse formation and killing of the target cell (49, 68, 161, 162). By detecting the active form of LFA-1, we could see an increased frequency of this form after cytokine stimulation. Similar data has previously been described following stimulation with target cells (48). Cytokine stimulation mainly affected non-educated cells to change their LFA-1 conformation while LFA-1 in educated NK cells was kept in tight control by the inhibitory receptors (Figure 3). In contrast, stimulation of NK cells with HLA class I negative target cells on the other hand resulted in higher frequency of educated cells expressing an active LFA-1 conformation.

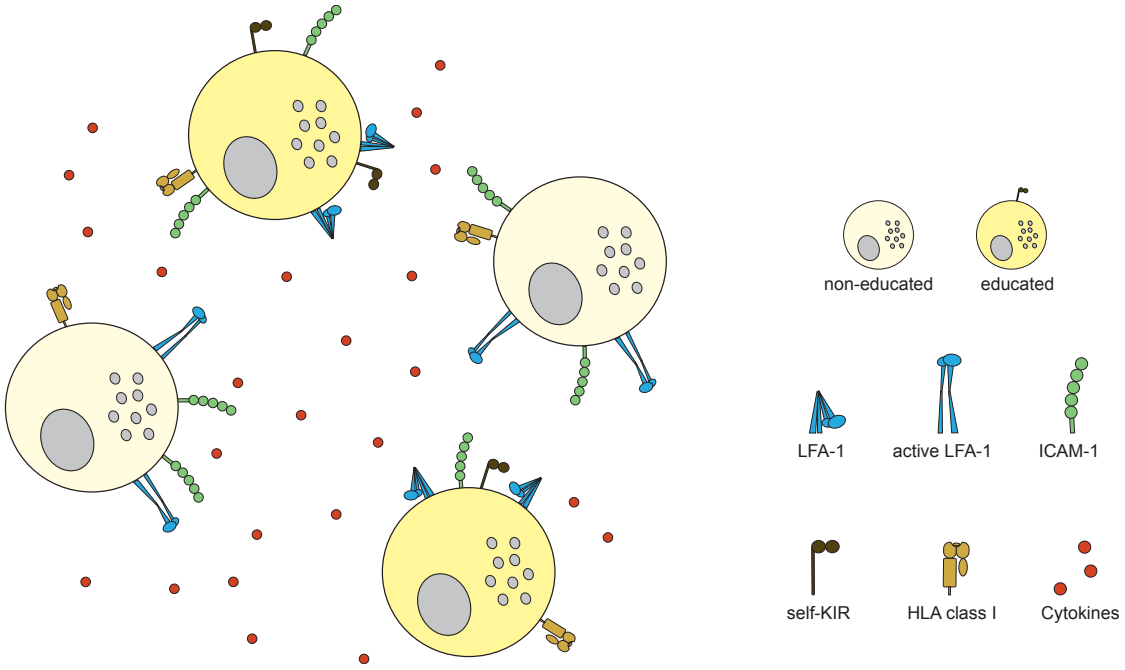

Figure 3: LFA-1 is activated mainly on non-educated NK cell upon cytokine stimulation, while LFA-1 on educated cells are kept in a closed inactive form by the inhibitory receptor interactions.

Studying the mobilisation of LFA-1 and DNAM-1 to the immunological synapse we found DNAM-1 to polarise even in the absence of its ligands on the target cell, although at a lesser degree compared to when the ligands were present. This recruitment suggests an outside-in signalling event enabling LFA-1 to activate and co-localise DNAM-1.

In an inhibitory immune synapse, KIR receptors binding to HLA class I accumulate in the middle, surrounded by LFA-1 molecules (163, 164). It is not known if DNAM-1 can be found in an inhibitory conjugate synapse or more peripheral. In stimulatory settings on lipid bilayer activating receptors have been shown segregated into different areas, 2B4 interacting at the centre while NKG2D in a peripheral region and co-localised with LFA-1 (165). Guia *et al* compared hypo-responsive to educated NK cells, and inhibitory receptors were found positioned in a cytoskeleton-based meshwork in both cases (156). The activating receptor NKp46 was found in similar meshwork as the inhibitory receptors in hypo-responsive cells, while in educated cells NKp46 was localised in signal-favouring, lipid dependent nanodomains and separated from inhibitory KIRs (156). In another study educated NK cells formed more conjugates and killing events compared to inhibitory receptor negative cells. This was not due to differences in LFA-1 itself but instead enhanced inside-out signals to LFA-1 by activating receptors, causing more stable adhesion in conjugate with the target cell (157). Conclusions from our studies suggests that DNAM-1 and LFA-1 mobilising at the immune synapse can contribute to the heighten effector functions in educated cells (Figure 4).

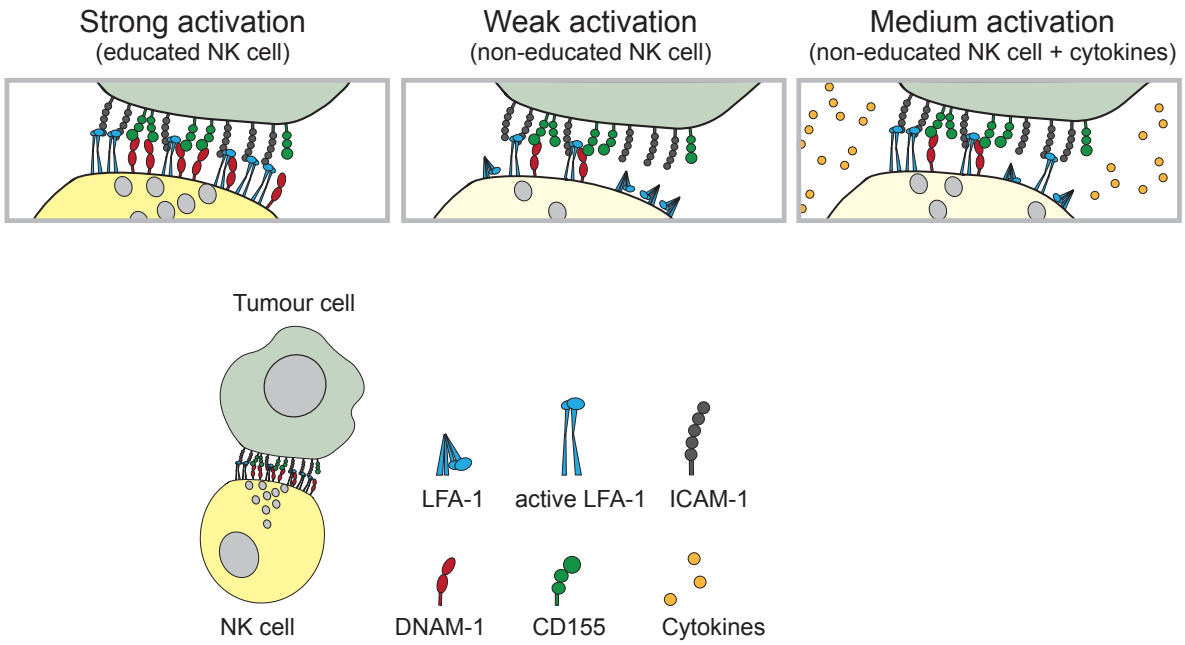

Figure 4: DNAM-1/LFA-1 at the immune synapse may contribute to strong activation in educated cells. Educated NK cells with high DNAM-1 expression facilitate strong activation including increase of active LFA-1. Non-educated cells have less DNAM-1 leading to less active LFA-1 and a weak NK cell activation. Non-educated cell pre-activated with cytokines have low DNAM-1 but increased active LFA-1 and can therefore respond better compared to non-educated NK cells without cytokine stimulation.

3.2 DEFECTIVE NK CELL CYTOTOXICITY RELATED TO DECREASED LEVELS OF ACTIVATING RECEPTORS

In **paper II** we examined the NK cells in patients suffering from MDS. Previous research on MDS patients had shown reduced function and in some cases decreased receptor expression in peripheral blood NK cells (166-169). One study linked the peripheral blood NK cell function with the severity of the disease (166). MDS mainly affect the bone marrow and because of this we set out to investigate the function and phenotype of NK cells residing in the bone marrow.

First, we examined the receptor expression on NK cells in MDS patients and age-matched healthy controls by flow cytometry. This revealed a decrease of two activating receptors on NK cells in MDS patients compared to controls, DNAM-1 and NKG2D. Since increased number of blasts in the bone marrow is associated with a worse clinical prognosis, we divided our cohort into patients with low or high frequencies. This stratification revealed a more distinct decrease of DNAM-1 and NKG2D in the patients with high blast counts (Figure 5).

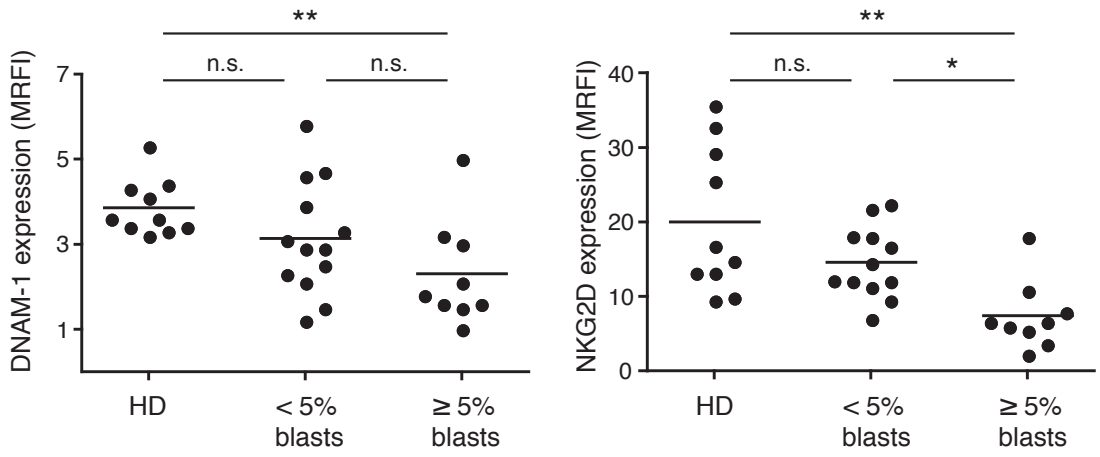

Figure 5: Reduced expression of DNAM-1 and NKG2D associated to high MDS blast frequencies. Receptor expression on bone marrow NK cells from healthy donors (HD) compared to MDS patients with low (<5%) or high (>5%) bone marrow blast count.

There are different mechanisms described for how NK cell receptor expression can be altered. In addition to physical receptor-ligand interactions leading to receptor internalisation as discussed above for DNAM-1, receptors may also be shed or degraded. Other factors that can influence the receptor expression are cytokines and tumour-derived factors such as microvesicles, soluble ligands and ROS. (146)

We examined the ligand repertoire on $CD34^+$ blast cells in MDS patients and found ligands for DNAM-1 and 2B4 to be frequently expressed whereas NKG2D ligands were less common. By comparing tumour cells and NK cells separated in transwells, previous work

from our laboratory showed that DNAM-1 receptor down-regulation in ovarian carcinoma was dependent upon physical interaction with the ligand CD155 (158). In another study, AML patients had decreased DNAM-1 levels associated with ligand expression on the AML blast cells (170). Similar events might also be the cause of reduced DNAM-1 and NKG2D levels on NK cells in the bone marrow of MDS patients, given the correlation we found between high blast counts and loss of these activating receptors.

Cytokines released in the tumour microenvironment can also affect NK cell receptor expression. TGF-β has previously been described to cause down-regulation of NKG2D expression on NK cells in MDS patients (171, 172). NKG2D has also been shown to be down-regulated due to ligand interactions (173-175). Furthermore, shed NKG2D-ligands may modulate the receptor expression and interfere with cytotoxic functions (176). In MDS patients, no soluble NKG2D-ligands have yet been reported, and in our study a minority of the patients had blast cells expressing NKG2D ligands. However, a recent study in mice (177) describes a more complex picture where tumour-associated macrophages desensitises NK cells through stimulating NKG2D by a membrane-bound ligand, while a soluble mouse NKG2D-ligand competes with this binding and counteract the functional impairment resulting in tumour growth inhibition. This mechanism to improve NK cell activity has to be further studied, but could potentially lead to new therapeutic strategies.

We also examined the NK cell function in **paper II** by studying degranulation after stimulating NK cells with tumour target cells, or by activating specific receptors through a reversed antibody-dependent cytotoxicity (ADCC) assay, in addition to measurements of cell death in tumour cells after being in contact with NK cells.

The degranulation of the patients' NK cells showed a defect function in both peripheral and bone marrow NK cells. Furthermore, by blocking specific NK cell receptors and studying tumour cell death after co-incubation with healthy donor NK cells, a major impact of the DNAM-1 receptor was detected for recognition and killing of MDS tumour cells (Figure 6). In these experiments HLA class I molecules were blocked to restrict the analysis to activating signals in the NK cell upon contact with the tumour cells. Finally, a comparison using autologous or allogeneic bone marrow-derived NK cells confirmed a defect in NK cells with poor cytotoxic response towards MDS tumour cells in patients.

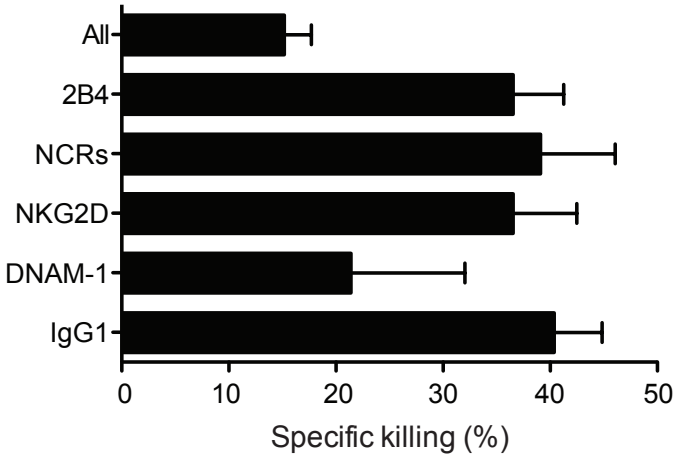

Figure 6: Blocking DNAM-1 receptor reduces killing of MDS blasts. Specific killing of bone marrow $CD34^+$ blast cells from a MDS patient. The NK cells from healthy donors were HLA class I blocked in addition to blockade of specific activating receptors as indicated.

Our results suggest an important role for the DNAM-1 receptor in recognition and killing of $CD34^+$ blast cells in MDS patients. Other studies of MDS patients have shown dysfunctional NK cells in peripheral blood (166-169), and in one case without any connection to changed NK cell receptor levels. This was instead related to a genetic alteration carried by a proportion of NK cells as well as the $CD34^+$ blast cell (168). MDS is a very heterogeneous disease and different mechanisms could be involved in the functional impairment of NK cells in peripheral blood and bone marrow. It is possible that for example also immunosuppressive cells releasing cytokines play a part in altering NK cell function.

3.3 ENHANCING NK CELL RECOGNITION BY SENSITISING TUMOUR CELLS

Modulating tumour cells is an attractive approach to increase the chance of cancer elimination by the immune system. In **paper III** we investigated the effect of treatment with selenite on NK cell tumour recognition. Selenoproteins have antioxidant properties and are thought to prevent cancer development at low doses, but have also been suggested able to inhibit carcinogenesis through induced changes in cell growth, signalling and apoptosis (178).

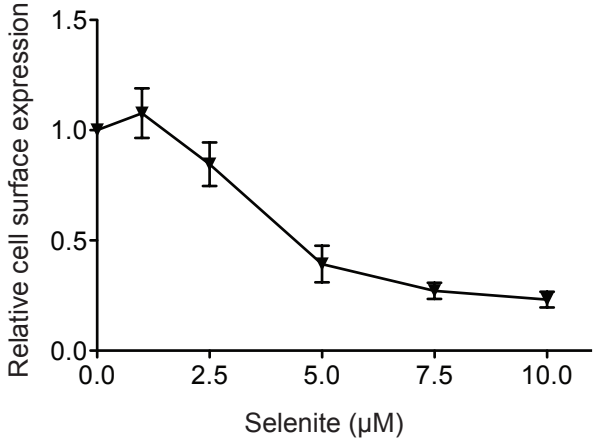

Figure 7: HLA-E expression decreased on tumour cells after selenite treatment. Cell surface level of HLA-E on STAV-AB tumour cell line, MFI expression compared to isotype control (N=7).

After treating a lung cancer cell line with selenite, NK cells degranulated at a higher level and also more efficiently killed the target cells. When studying the tumour cells in detail it became evident that two surface molecules recognised by NK cells were altered, MIC/A and HLA-E (Figure 7). MIC/A binds to the activating NK cell receptor NKG2D, and thus its down regulation is unlikely involved in triggering increased NK cell cytotoxicity. HLA-E on the other hand, can bind both to the inhibitory receptor NKG2A/CD94 and the activating receptor NKG2C/CD94. NKG2A/CD94 is expressed on up to 70-80% of human NK cells and a decrease in HLA-E levels would therefore influence the behaviour of a large population of NK cells. In our experimental setting we confirmed $NKG2A^+$ NK cells selectively showed increased recognition of selenite exposed tumour cells. Measuring total protein and mRNA levels showed selenite-induced inhibition of HLA-E expression at a posttranscriptional level, and that this was further associated to oxidative stress in the tumour cells.

ROS are important in cell signal transduction with roles in differentiation, migration, proliferation and apoptosis (179). Hence, oxidative stress can both promote and supress cancer progression and the cellular levels of ROS are tightly controlled by antioxidant systems. Tumour cells often display increased ROS and antioxidant mechanisms contributing to cancer progression and avoidance of apoptosis. Increasing oxidative stress can therefore specifically target the malignant cells and tip the balance towards uncontrollable ROS levels and cell death. On the other hand, if the increased ROS is still manageable it might instead enhance tumour growth. Treatment with compounds such as selenite would therefore require careful evaluation of dose levels.

The HLA-E molecule is transcribed in the majority of cell types in humans, while cell surface expression normally is limited to immune cells and endothelial cells (180). However, various cancer cells have been reported to over-express HLA-E, e.g. lymphomas, gliomas and melanomas (181-183). This could be a specific mechanism to evade NK cell cytotoxicity, or reflect a total increase in HLA class I molecules and escape of T cell recognition. Oxidative stress caused by selenite may reduce HLA-E levels through general protein reduction, prioritising synthesis of cell survival associated proteins. Although promising as cancer treatment, further studies are needed to understand the impact of dose levels and the overall advantage of selenite administration, as well as the detailed mechanism for HLA-E down-regulation in tumour cells.

3.4 NK CELL RESPONSES DURING CANCER TREATMENT

In **paper IV** we studied the longitudinal effect on NK cells in follicular lymphoma patients treated with monoclonal antibodies. We collected peripheral blood and tumour lymph node fine-needle biopsies from previously untreated patients. The samples were collected weekly before each first four doses of rituximab (Figure 8).

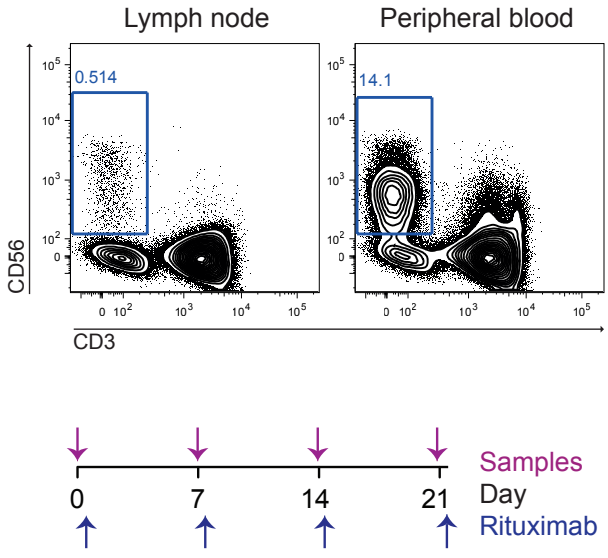

Figure 8: Tumour lymph node and peripheral blood NK cells from follicular lymphoma patient. Samples were collected weekly before each rituximab treatment.

We found that the NK cell frequency in tumour lymph node (LN) samples was low and in the same range as in healthy tonsils. Previous studies have reported NK cells in healthy tonsils and lymph nodes to mainly constitute of $CD56^{bright}$ NK cells being CD16 and KIR negative (184, 185). The phenotype of the LN-NK cells in our study were to a higher degree $CD56^{dim}$ NK cells compared to tonsil controls, furthermore expressing intermediate levels of activating and inhibitory receptors and maturation and cytotoxic markers as CD16, KIRs, CD57, granzyme A/B and perforin, compared to tonsils and peripheral blood (PB) NK cells. This could be due to an on-going immune response towards the proliferating tumour cells.

In PB we observed a drop in frequency and absolute counts of NK cells after the first rituximab administration, confirming previously reported observations (186). NK cells are recruited to lymph nodes undergoing an immune response in mice, in a CXCR3 and L-selectin-dependant manner (187, 188). It is unknown if similar events takes place in humans although it is believed that NK cells contribute to the elimination of malignant B-cells in the lymph node. We could not detect an overall influx of LN-NK cells, but it is possible that by collecting samples weekly we missed important early kinetics. The longitudinal phenotype of both LN-NK and PB-NK cells were stable over time regarding CD57, KIRs, CD16, granzyme A and perforin. We noted a small increase of NKG2A expression in PB-NK and an increase of granzyme B in LN-NK cells.

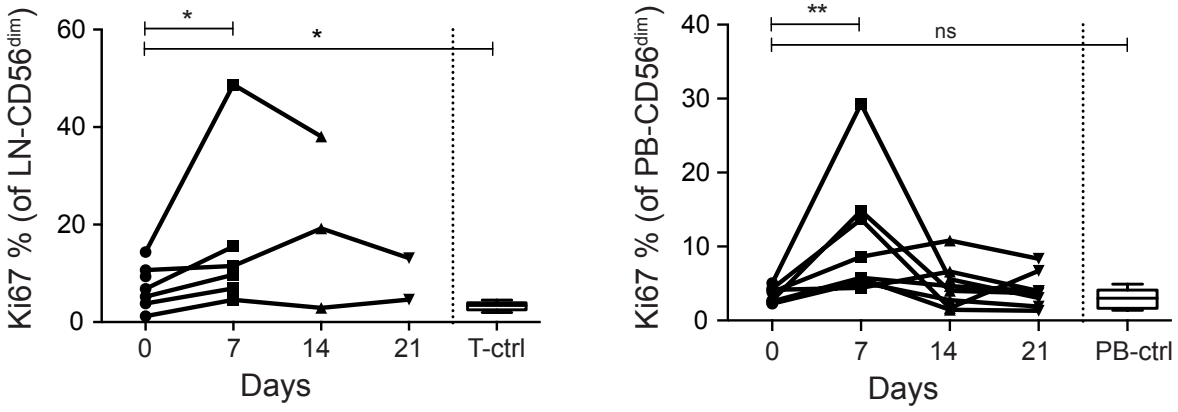

Figure 9: Proliferative increase in lymph node and peripheral blood NK cells after rituximab treatment. Ki67 expression in NK cells from patients before and after treatment, and compared to healthy control tonsils (T-ctrl) and peripheral blood (PB-ctrl) (Patients N=8, Ctrl N=8).

Ki67 is an intracellular transcription factor found in recently divided or cycling cells (189). Staining for this activation marker we found it to be expressed at higher frequencies in LN-NK cells before treatment than in control tonsil NK cells, while the PB-NK cells had comparable levels as healthy control PB-NK cells. After onset of treatment, we detected a consistent increase in Ki67 expression in both LN-NK and PB-NK cells (Figure 9). Further analysis of $Ki67^{+}$ LN-NK cells was not possible due to low cell number. In proliferating PB-NK cells we detected a slightly more differentiated phenotype at day 7 compared to day 0

with an increase in CD16, CD57, KIRs, granzyme A and perforin expression. A recent study from Rosario *et al* found enhanced NK cell responses towards lymphoma cells by combining rituximab treatment with an IL-15 superagonist, ALT-803 (190). Studying this effect with improved killing capacity in mice and *in vitro*, they furthermore detected increased granzyme B and perforin expression in NK cells. Additionally, long-term activation of the IL-15 receptor with ALT-803 induced proliferation of $CD56^{bright}CD16^{+}$ NK cells with a maintained ADCC function (190). It would be interesting to evaluate to what degree the NK cell proliferation we detected in FL patients on rituximab monotherapy is linked to endogenous cytokine production, and moreover if the combination of IL-15 and rituximab treatment can improve anti-lymphoma responses and survival also in humans.

We assessed the function of PB-NK cells by re-stimulating cells *in vitro* with a HLA class I negative B-cell line coated with rituximab. After co-incubation the NK cells were stained for a degranulation marker (CD107a) and cytokine production (IFNγ). Comparing the responding NK cells revealed a decrease in cytokine production in the majority of the patients seven days after the first rituximab administration. This altered functionality was moreover associated to the proliferative increase, possibly indicating that the onset of cell division reprogram the functionality leading to decreased cytokine production. Hyporesponsiveness in NK cells following treatment with rituximab has been reported *in vitro* (191, 192). In these short-term models, cytotoxicity was decreased and in one of the studies the NK cell function could be restored after 2 days in IL-2. In contrast, our data showed altered cytokine production while the degranulation towards rituximab-coated targets was less affected. Furthermore we found stable or increased levels of the cytotoxic effector molecules granzyme A/B and perforin after treatment, suggesting the ability to perform cellular cytotoxicity remained intact. The decreased functionality we observe may represent a functional exhaustion following the primary *in vivo* response to rituximab therapy, and if so the variation in the *in vitro* response may hold clues to the efficacy of the therapy.

A few patients showed an opposite trend with a more stable or increased NK cell function at day 7 and beyond. These patients had a weaker proliferative increase after treatment and presence of adaptive NK cell subsets expressing NKG2C and CD57. Adaptive NK cells have an increased capacity to produce IFNγ, caused by specific demethylation in the IFNγ promoter frame in these cells (31, 35-37). In our study, patients having $NKG2C^{+}$ NK cells could theoretically thus already from start be better equipped for cytokine production in response to rituximab treatment and explain the functional stability over time.

Larger studies has to be done in order to link the clinical response to variation in immune repertoires, but the *in vivo* and *in vitro* responses we observed here may give some indications regarding the role of the NK cells during rituximab therapy.

4 CONCLUSIONS

Paper I

- The expression level of DNAM-1 correlates with the education status of NK cells
- LFA-1 is activated mainly on non-educated NK cell upon cytokine stimulation, while inhibitory interactions maintain LFA-1 in an inactive form on educated NK cells
- Upon contact with target cells, LFA-1 and DNAM-1 is recruited to the immunological synapse

Paper II

- NK cells in the bone marrow of MDS patients display reduced expression of DNAM-1 and NKG2D. The degree of receptor downregulation correlates with the frequency of malignant blasts in the bone marrow
- DNAM-1-CD155/CD122 interactions have an important role in NK cell-mediated killing of MDS blasts
- NK cells from MDS patients are functional hypo-responsive and display poor ability to recognise $CD34^+$ MDS blasts

Paper III

- NK cell-mediated recognition and killing of a mesothelioma tumour cells line are increased following pre-treatment of the tumour cells with selenite
- Selenite induced a dose-dependant decrease in HLA-E expression on mesothelioma tumour cells, caused by oxidative stress-induced protein reduction at a posttranscriptional level, triggering activation of $NKG2A^+$ NK cells

Paper IV

- NK cells in FL tumour lymph node have an intermediate maturation grade compared to the immature healthy lymphoid tissue and mature NK cells in peripheral blood
- NK cells exposed to rituximab display high expression of Ki67, indicative of high proliferate activity *in vivo* one week after treatment, both in lymphoid tissue and peripheral blood
- NK cells in peripheral blood from rituximab-treated FL-patients have an altered cytokine production upon *in vitro* re-stimulation with rituximab-coated targets

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